REMARKS

These remarks are in response to the Final Office Action mailed February 27, 2007. Claims 16 and 17 have been amended. No new matter has been introduced by the filing of this paper.

I. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Claims 9-16 stand rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for predicting the inhibitory action of alcohols on cytochrome P-450 aniline p-hydroxylation and perhaps some other properties of alcohols or simple organic molecules, such as vapor pressure, allegedly does not reasonably provide enablement for predicting or determining the specific activity, chemical or physical property or function of compounds other than alcohols. Specifically, the Examiner alleges that Applicants have not established a correlation between sensor fingerprints and nucleotide or protein sequences. Applicants respectfully traverse this rejection.

The Final Office Action alleges that "assuming arguendo that Drummond supports Applicants' position as alleged in content, it [Drummond et al.] is unpersuasive in traversing the scope of enablement rejection because it does not appear to show what was well known in the art at the time of the invention." (see Page 3 of the Final Office Action). Attached hereto as Appendix A is a reference identifying a number of DNA sensors known in the art at the time of Applicants' invention.

It appears to Applicants that the Examiner is viewing the disclosure and evidence of record, not as a whole, but by selectively considering pieces of the

disclosure and evidence in a void (e.g., whether there are DNA sensors, whether there are mass sensors, whether there are various transduction modalities). Applicants respectfully submit that the data (both in the specification and subsequent references) demonstrate:

1) Biological molecules such as DNA can be measured by sensors (e.g., Drummond et al.; Junhui et al.);
2) Other references demonstrate properties including mass can be measured. For example, Ballantine (see below) teaches that mass can be measured by sensors (see, e.g., page 11 of the Office Action);
3) Molecular characteristics such as functional groups, hydrogen bonds and chirality can be measured by sensors (specification and subsequent reference);
4) That different sensor types and modalities exist for measuring properties;
5) Post filing reference including issued patents demonstrate analyte properties can predicted using sensors. For example, solubility (see, e.g., U.S. Patent No. 7,117,102), thermal stability and dielectric properties (see, e.g., U.S. Patent No. 6,668,230) and other physical chemical properties (see, e.g., U.S. Patent Nos. 6,625,548; 6,553,318; 6,535,824; 6,535,822; and 6,438,497).

The Office Action goes on to ask, "...do Drummond and the US patents listed by Applicants in Applicants' view mean that their invention is obvious?" Applicants submit that Drummond is after Applicants' filing date and thus is not prior art. Furthermore, the Examiner's pending rejections are questioning whether DNA sensors exist, whether DNA sensor exist does not render the claimed invention obvious as the claimed invention has to be viewed as a whole and not picked apart. Furthermore, arriving at an obviousness rejection would be based upon hindsight.

The Office Action at page 5, bridging to page 6 alleges that Applicants have only demonstrated a homogenous/identical sensor array. However, the sensors used are in fact different sensors in that they comprise different materials that respond differently to an analyte (see, e.g., Figure 1A and Figure 5).

Furthermore, the Office Action alleges that the disclosure does not describe "mass sensors". Applicants submit that the term "mass sensor" is being coined by the Examiner to refer to sensor that measure mass. As is known in the art, resonance mechanic frequency sensors can sense a mass or change in mass and thus, should the Examiner wish to refer to these as "mass sensors" such sensors are disclosed in the application (see, e.g., paragraph [0006]).

Applicants respectfully submit that when the claims are viewed in combination with the teachings of the specification and the knowledge of those of skill in the art at the time the invention was made and the evidence provided in the record, the claims are fully supported and enabled for their scope.

It is important to remember that amino acids/proteins/nucleic acids are chemical compounds. The Examiner has acknowledged that the Applicants have demonstrated alcohol and numerous other chemical compounds can be sensed and analyzed by the invention. Nucleic acids and proteins are chemical compounds comprised of chemical bonds and side groups. Applicants respectfully submit that it has established reasonable correlation between sensor fingerprints and nucleic acids or proteins by providing arguments and reasoning, documentary evidence, and combinations thereof demonstrating that chemical entities can be predicted, complex polymer characteristics can be predicted such as solubility (see, e.g., U.S. Patent No. 7,117,102), thermal stability can be predicted and complex dielectric properties (see, e.g., U.S. Patent No. 6,668,230).

The invention has been demonstrated to recognize a plurality of analyte properties. Accordingly, limiting to a particular property narrows the scope of the

claims in view of a broader disclosure supported by the specification and data of record.

The Examiner also appears to be concerned that the scope of the claims is unbounded as to the type of sensor array. However, the Examiner has acknowledged that numerous sensor types and sensing modalities are known in the art (see, e.g., paragraph 0016 of the specification and page 6, last paragraph of the Final Office Action mailed October 26, 2005). Applicants (as well as the Examiner) have provided evidence that other sensor types work to measure analyte properties. Applicants submit that to limit the type of sensor would limit the use of the claimed invention in view of a broader disclosure. The different types of sensor provide different information relative to identifying an analyte. For example, if the sensor array contained only a single type of sensor, only a single interaction of an analyte with the sensor would be probed and reported, thus the profile of data obtained would be limited and thus the ability to "fingerprint" a molecule would be limited.

Applicants' specification, the Examiner's admissions, and corroborating evidence of sensor modalities (e.g., transducer-types and analyte-types) support Applicants' invention. Accordingly, Applicants respectfully request withdrawal of the rejection.

II. REJECTION UNDER 35 U.S.C. §103

Claims 11, 16, and 17 stand rejected under 35 U.S.C. §103(a) because while allegedly not enabled as set forth above, the invention *is enabled* and allegedly obvious in view of Ballantine Jr. et al., ("Correlation of Surface Acoustic Wave Device Coating Response With Solubility Properties And Chemical Structure Using Pattern

Recognition," Anal. Chem. 88:3058-3066, 1986; "Ballantine"). Applicants respectfully traverse this rejection.

As the Examiner correctly points out, Ballantine does not teach or suggest the following elements:

> . . . not including the analyte of interest, identifying the activity, chemical or physical property, or function of at least one known analyte that most closely correlates with the sensor array signal profile, wherein the correlation of the sensor array signal profiles to the previously obtained signal profiles is predictive of a specific activity, chemical or physical property, or function of the analyte of interest. . .

(as recited in Applicants' independent claims). Furthermore, in the *two decades* that have since passed between publication of Ballantine and today, no party has published or described the elements of Applicants' claimed invention. Furthermore, as the Examiner will notice current techniques utilize training samples that include the analyte of interest as part of the training set, this is drastically different than Applicants' claimed invention.

For at least the foregoing reasons, Applicants submit that the claimed invention is non-obvious over the Ballantine reference. Accordingly, Applicants respectfully request withdrawal of the rejection.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY LLP

/s/ Joseph R. Baker, Jr.

Date: August 27, 2007 By: _____

Joseph R. Baker, Jr.
Registration No. 40,900

P.O. Box 1404
Alexandria, VA 22313-1404
858.509.7300

APPENDIX A

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DNA BASED BIOSENSORS

ZHAI JUNHUI, CUI HONG AND YANG RUIFU

Institute of Microbiology and Epidemiology, 20 Dongdajie, Fengtai District Beijing 100071, P.R. China

ABSTRACT

Compared to advances in enzyme sensors, immunosensors, and microbial biosensors, relatively little work exists on DNA based biosensors. Here we review the DNA based biosensors that rely on nucleic acid hybridization. Major types DNA biosensors—electrochemical, optical, acoustic, and piezoelectric—are introduced and compared. The specificity and response characteristics of DNA biosensors are discussed. Overall, a promising future is foreseen for the DNA based sensor technology.

KEY WORDS

DNA biosensor, biosensors, nucleic acid hybridization.

INTRODUCTION

Since the first enzyme electrode was reported over three decades ago [1], biosensor technology has advanced exponentially [2]. The research effort in biosensors has certainly been matched by a burgeoning literature, with many excellent general reviews [3,4] and monographs [5–8] now available. Recently, a handbook of chemical and biological sensors has also appeared [9].

A biosensor by definition is an analytical device that combines the specificity of a biological sensing element with a transducer to produce a signal proportional to target analyte concentration. This signal can result from a change in proton concentration, release or uptake of gases such as ammonia or oxygen, light emission, absorption or reflectance, heat emission, mass change, etc. The signal is converted by a transducer into a measurable response such as current, potential, temperature change, absorption of light, or mass increase through electrochemical, thermal, optical, or piezoelectric means. The signal may be further amplified, processed, or stored for later analysis. In principle, any receptor may be combined with any suitable transducer to produce an operational biosensor [4].

Work on biosensors has focused mostly on enzyme sensors [10–12], immunosensors [13–15], and microbial sensors [16,17]. Studies of DNA biosensors are relatively rare [2]. In recent reviews, Mulchandani and Bassi [4] and Vadgama and Grump [3] noted DNA as a biological recognition element for biosensors, but the accomplishments of DNA biosensor technology were not mentioned.

Deoxyribonucleic acids (DNA) are arguably the most important of all biomolecules. The unique complementary structure of DNA between the base pairs adenine/thymine and cytosine/guanine has been the basis for genetic analysis over the last few decades. The ability of a single stranded DNA (ssDNA) molecule to 'seek out', or hybridize to, it's complementary strand in a sample is the foundation of DNA-based detection systems. There is a great potential market for simple, cheap, rapid, and quantitative detection of specific genes. Areas of application include clinical, veterinary, medico-legal, environmental, and the food industry.

PRINCIPLES OF THE DNA BIOSENSOR

The DNA techniques, including hybridization, amplification, and recombination, are all based on the double helix structure of the DNA. Nucleic acid hybridization is the underlying principle of DNA biosensors. In 1953, Watson and Crick described the structure of DNA and the role of the DNA molecule in holding the information for cell reproduction and function [18]. DNA is composed of four repeating nucleotides (sometimes called nucleotide bases or simply bases): adenine, guanine, cytosine, and thymine. DNA is coiled to form a double helix (double-stranded DNA, or dsDNA) composed of two strands held together by hydrogen bonds that can be broken by heat or high pH. The single stranded DNA (ssDNA) is relatively stable, but on removal of the heat source or pH extreme, the DNA molecule will re-form (reanneal) into the double stranded configuration. Reannealing between the ssDNAs from different sources is called hybridization. The reannealing of the dsDNA is possible because nucleotide bases will re-form hydrogen bonds only with specific complementary bases: adenine pairs with thymine, and cytosine pairs with guanine. In RNA, the nucleotide base uracil replaces thymine and pairs with adenine. The stability of the hybridization depends on the nucleotide sequences of both strands. A perfect match in the sequence of nucleotides produces very stable dsDNA, whereas one or more base mismatches impart increasing instability that can lead to weak hybridization of strands. The same basic principles apply to RNAs, the transcriptional products of cell growth and reproduction. RNA-RNA and RNA-DNA hybridizations can occur.

Like other biosensors, DNA sensors are usually in the form of electrodes, chips, and crystals; hence, hybridization on a sensory surface is a solid-phase reaction. Solution hybridization is more rapid than hybridization on solid-supports, but unless the assay is a

homogeneous assay, a separation step is required before final detection [19,20]. Solid phase or filter hybridization is the longest established and the most often used [21].

The kinetics of nucleic acid hybridization at a solid substrate-solution interface are still not well understood because there is a dearth of suitable methods for the continuous measurement of the hybridization process. The process is also difficult to predict from theoretical considerations, partly because the exact concentration of the immobilized nucleic acid and its availability for hybridization are unknown [22]. Furthermore, the actual mechanism of strand association in solid-support hybridization remains obscure.

The kinetics and mechanism of nucleic acid single strand hybridization have been widely studied only in cases where both strands are in free solution [23]. Hybridization in solution is believed to be a two-step process involving nucleation and zippering up. Nucleation is the rate-limiting step, and a second order reaction equation can describe the process. The nature of the hybridization reaction on solid surfaces has been assumed to approximate closely to that of hybridization in solution. However, the rate of solid-phase hybridization is only about a tenth to a hundredth of that in solution [24]. Although not systematically examined, it has been suggested that efficient hybridization to DNA attached to solid supports can be impeded by several related phenomena. For example, the immobilized DNA molecules may link to solid surface at several points along the DNA chain, hence some of the attached DNA may not be accessible for hybridization [25].

Lack of a mechanistic knowledge notwithstanding, a variety of DNA based biosensors are now available for research as noted in Table 1. Some of the main types are discussed in depth in the following sections.

DNA BIOSENSORS BASED ON OPTICAL TRANSDUCTION

Several kinds of optical transducers can form the basis of a DNA biosensor. Such transducers include fluorescence [26–28], surface plasmon resonance [29–31,40], and Raman spectroscope [32] as discussed separately below.

Fiber Optics

The fluorescent DNA stain ethidium bromide (EB) is a commonly used dye for the detection of DNA [41–43]. The fluorescent ethidium cation (3,8-diamino-6-phenyl-5-ethyl-phenanthridium) strongly associates with dsDNA by intercalation into the base stacking region and, in some cases, the major groove of the double helical structure [41]. EB has an absorption maximum of 510 nm; detection of dsDNA is achieved by exposing it to an EB solution, washing off the unbound EB, and measuring the fluorescence intensity by UV-visible spectrometry. The fluorescence intensity is directly proportional to the amount of EB-intercalated DNA. Biosensors can be designed to use this principle.

Table 1. Types of DNA biosensors.

Type	Biological Element	Transducer	Reference
Optical	DNA		
Fiber optics		Optical fiber	Piunno *et al.* [26] Piunno *et al.* [27] Hirschfeld [28]
Surface plasmon resonance		Resonant mirror	Watts *et al.* [29]
Biomolecular interaction analysis		BIAcore	Nilsson *et al.* [30] Wood [31]
Raman spectroscopy		SERG probes	Vo-Dinh *et al.* [32]
Electrochemical	DNA	Carbon paste electrodes	Millan *et al.* [33–35]
Piezoelectric	DNA		
Frequency		Crystals	Campbell *et al.* [36] Okahata *et al.* [37]
Acoustics		Crystals	Su *et al.* [25,38,39]

One of the first biosensors for direct analysis of DNA hybridization by use of an optical fiber was developed by Piunno *et al.* [26]. Single stranded ssDNA thymidylic acid icosanucleotides (dT_{20}) were grown onto optical fibers. The fibers were first derivatized with γ-aminopropyltriethoxysilane (APTES) onto which a spacer arm of 1,10-decanediol bis-succinate terminated with 5'-O-dimethoxytrityl-2'-deoxythymidine was covalently attached. The synthetic route used to grow the ssDNA was the well established solid-phase phosphoramidite methodology. The covalently immobilized oligomers were able to hybridize with available complementary ssDNA (cDNA) which was introduced into the local environment to form dsDNA. The hybridization event was detected by the use of the fluorescent EB stain. The sampling configuration utilized total internal reflection within the fiber, resulting in an intrinsic mode optical sensor. The non-optimized procedure used standard hybridization assay techniques to provide a detection limit of 86 $ng \cdot mL^{-1}$ cDNA, a sensitivity of 83 % fluorescence intensity increase per 100 $ng \cdot mL^{-1}$ of cDNA initially

present, and a hybridization analysis time of 46 min. The sensor remained active after prolonged storage (3 months) and severe cleaning conditions (sonication).

Future work is likely to use fluorescent probes covalently immobilized directly onto immobilized ssDNA via 5' derivatization. Such probes would have high quantum yields when intercalated into the base stacking region of dsDNA and near zero quantum yields in the presence of immobilized ssDNA. The tethered fluorescent probe should provide a reduction in the response time, be unaffected by non-specific absorption, and offer greater portability because of the reduced demand for external solution treatment. Washing with hot chaotropic salt solutions is also being investigated for removing cDNA strands from the surface of the fiber. Chaotropic salts decrease duplex stability and, hence, should improve the regenerability of the biosensor. This work is now being extended to detection of pathogenic bacteria [26, 27].

Although EB is often used for visible detection of DNA, under ideal conditions it has a sensitivity of 5 to 50 ng DNA which is not particularly high compared with polymerase chain reaction (PCR) or nucleic acid hybridization assays. At the same time, EB is notoriously harmful. So far no effective replacement for EB is available, but promising substances are under investigation.

Resonant Mirror

Optical methods, such as those using surface plasmon resonance (SPR) and monomode dielectric wave guides, have been extensively developed in recent years. The resonant mirror is an evanescent wave sensor which has been designed to combine the simple construction of SPR devices with the enhanced sensitivity of wave guiding devices. Detection of enzyme-substrate [44], antibody-antigen [44], and protein-cell interactions [29] by the resonant mirror device has been reported. This technology has recently been applied to direct and rapid detection of DNA-DNA hybridization [29]. Biotinylated oligonucleotide probes were immobilized on the sensor's surface, via streptavidin, and hybridization of a complementary target oligonucleotide (40-mer) was monitored in real time. The interaction at the sensory surface was shown to be sequence specific under conditions of low stringency. Regeneration of the surface-immobilized probe was possible, allowing reuse without a significant loss of hybridization activity. A comparison of probes indicated that the relative position of complementary sequence and the length of probe affected the hybridization response obtained. The potential of the sensor for quantitation of a hybridized DNA target was investigated. From radiolabelling data, the lowest amount of hybridized target sequence which could be determined directly was 19.9 fmol·mm^{-2} (263 pg·mm^{-2}) of sensor's surface. The dependence of the sensor's response on the concentration of probe and target oligonucleotide was established. Utilizing the assay as an end-point determination method,

the lowest detectable concentration of target oligonucleotide (40 mer) was 9.2 nM which compared favorably with methods described previously.

Raman Spectroscopy

Raman spectroscopy is a useful tool for chemical analysis because of its excellent capability for chemical group identification. One limitation of conventional Raman spectroscopy is its low sensitivity; hence, effective measurements often require powerful and costly laser sources for excitation. However, this method has attracted renewed attention because of the recent observation that Raman scattering efficiency can be enhanced by factors of up to 10^8 when a compound is adsorbed on or near special metal surfaces [45]. This modified technique is known as surface-enhanced Raman scattering (SERS) spectroscopy. In 1994, Vo-Dinh *et al.* [32] reported the development of a new type of DNA probe based on surface-enhanced Raman scattering detection. These surface-enhanced Raman gene (SERG) probes do not require the use of radioactive labels, while having a great potential for sensitivity and selectivity. In the work reported [32], nitrocellulose filters containing the DNA to be hybridized were placed in siliconized 1.5 mL microfuge tubes. Distilled water was added and brought to boil on a water bath. The water was then removed by gentle aspiration. Negative controls consisted of labeled DNA that was not complimentary to the immobilized DNA. The filters were incubated overnight at 40°C in hybridization solutions containing 2 $ng \cdot mL^{-1}$ of the labeled probe [32]. DNA which did not hybridize was removed by washing three times with SSPE-20X solution containing 0.1 % SDS at room temperature. Results showed that the SERG probe could have a wide variety of applications in nucleic acid identification. Thus, single strands of DNA fragments can be labeled to SERS probes. The resulting SERG probes may be used to identify genes or detect bacterial and viral components. Use in PCR is also feasible. However, this hybrid chemical-biological detection methodology requires expensive instrumentation and it is slow. Its use in a biosensor configuration is unlikely.

Biomolecular Interaction Analysis

Developments in instrumentation for biomolecular interaction analysis (BIA) using biosensor technology have made real-time monitoring biological events possible [30]. The biosensor often used, BIAcore (Pharmacia Biosensor), is based on surface plasmon resonance (SPR) for detection of changes in refractive index over time at the sensory surface. These changes are proportional to the mass of molecules bound to the surface, and are shown in a 'sensorgram' as resonance units (RU) plotted against time. From the results, stoichiometric and kinetic data for the interaction can be determined. BIA has been used primarily to study protein-protein interactions, e.g., antibody-antigen recognition [15], and to a lesser extent to study DNA-protein interactions and DNA-DNA hybridizations [30,31]. In

Wood's work [31] a reaction surface was prepared in BIAcore by covalent coupling of a ligand to the derivatized dextran matrix located on a sensor chip surface. Four areas of the chip surface were used as unique reaction chambers. Each chamber was individually fed by a fluid channel. Although primary ligands were bound to the dextran matrix, reaction proceeded in a liquid environment. Microliter amounts of reactants were delivered by operator-programmed software. Reaction surfaces, bound to a 50-nm gold surface, were mechanically brought in contact with the glass interface of the optical system. Monochromatic light, deflected at the reaction surface, was reflected as a function of the difference in refractive indices of the glass and liquid layer. A part of the resulting total internal reflectance of light was monitored as the reaction occurred. Concomitant to reflectance, evanescent wave energy was generated within the metal surface. This change was monitored electronically using a separate diode array for each of the four channels and recorded graphically as reactions occurred. A response, expressed as RU, of 100 roughly corresponded to 1 % per mm^2 monoclonal antibody (mol wt 100,000-120,000 daltons). Response may vary with the protein used [31]. The hybridization reaction was carried out at room temperature and positive signals were obtained within 7 min. In addition, biotinylated and complementary DNA oligonucleotides were prehybridized prior to injection. Hybrid pairs were captured by the avidin surface giving results commensurate with the concentrations injected. Control experiments showed that identically sized, but dissimilar DNA oligonucleotides did not hybridize with the biotinylated base probe. Nonspecific attachment of DNA directly to the reaction surface was not observed. This was the first account of real-time detection of specific hybridization within a fluid flow system in less than 10 min at room temperature [31].

Nilsson *et al.* [30] boosted this technology further, not only for DNA hybridization kinetics, but also for DNA strand separation and enzymatic modification. DNA fragments were immobilized onto a biosensor surface using the high-affinity streptavidin-biotin system and subsequently used to monitor different unit operations in molecular biology, e.g., DNA strand separation, DNA hybridization kinetics, and enzymatic modifications. A model system comprising six oligonucleotides was used; the oligonucleotides could be assembled into a 69-bp double-stranded DNA fragment. Using this system, the biosensor approach was employed to analyze multistep solid-phase gene assembly and the performance of different enzymes routinely used for the synthesis and manipulation of DNA. In addition, a concept for the determination of single-point mutations in DNA samples was described. All these DNA manipulations were linked to value changes of RU. For example, for oligonucleotide hybridization, the baseline of RU was about 15,100 (no hybridization); 140 s after hybridization solution was injected onto the sensor's surface, the RU rose to 17,400 and maintained this value for at least 500 s.

There exists the possibility of using 'real' samples for DNA hybridization reactions with BIAcore. Already, the ability to target portions of DNA responsible for defined factors using probes and monoclonal antibodies as tools, is patently evident. Although many variations in hybridization procedures exist, none are as simple and straightforward as those presented by Nilsson *et al.* [30]. Nevertheless, specific hybridization occurred in less than 10 min within a flow stream at room temperature. The supporting evidence of specificity, as shown by lack of nonspecific binding to the avidin layer, and no interaction of an identically sized but dissimilar DNA oligonucleotide, reinforced the validity of the method. In addition, capture of prehybridized oligos at differing concentrations of DNA gave responses commensurate with the respective concentrations [30].

Real-time BIA is a powerful technique that derives its versatility from the ability to monitor interactions through changes in mass concentration at the sensor's surface, with no analyte labeling. The interaction is followed in real time, making BIA an ideal technique for measuring interaction kinetics which are frequently difficult to analyze with other methods. However, BIA can also be used to determine binding specificity and analyte concentration, and the simplicity of the technique can provide advantages even for applications where conventional methods are well established.

DNA BIOSENSORS BASED ON ELECTROCHEMICAL TRANSDUCTION

Millan *et al.* [33] attempted development of a DNA sequence-selective biosensor employing electrochemical transduction. A DNA probe sequence was covalently bound to the surface of an amperometric electrode [33–35]. Hybridization of the immobilized probe sequence with its dissolved complement produced an immobilized double strand that could be detected using redox-active metal/polypyridine complexes that associate selectively and reversibly with double-stranded DNA. The presence of the immobilized double strand caused preconcentration of the metal complex in the DNA layer near the surface of the electrode, and it resulted in much larger voltammetric peak currents than are observed for immobilized single-stranded DNA. The authors studied the complexes tris(1,10-phenanthroline)cobalt (III) perchlorate and tris(2,2'-bipyridyl)cobalt (III) perchlorate, which are reversibly reducible to their cobalt (II) forms with formal potentials of 0.317 V and 0.085 V vs SCE, respectively, well within the window of +1.2 V to −0.9 V vs SCE over which DNA is electroinactive. Studies with oxidized glassy carbon electrodes have shown that oligo- and polydeoxynucleotides can be covalently immobilized onto carboxylic acid groups using water-soluble carbodiimide and *N*-hydroxysuccinimide coupling reagents and that this reaction is selective for immobilization through deoxyguanosine residues [34].

Carbon paste electrodes modified by inclusion of either octadecylamine or stearic acid were used as solid phases to which DNA was covalently bound. Immobilized DNA was detected by voltammetry of solutions containing submillimolar quantities of

$Co(bpy)_3(ClO_4)_3$, $Co(phen)_3(ClO_4)_3$, and $Os(bpy)_3$-Cl_2 (bpy = 2,2'-bipyridine; phen = 1,10-phenanthroline), all of which associate reversibly with immobilized DNA and yield increased peak currents at DNA-modified electrodes. Immobilization onto octadecylamine-modified electrodes was performed using a water-soluble carbodiimide, and at high DNA concentrations in the surface. Optimization of the deoxyguanosine- (dG-) selective immobilization reaction for stearic acid-modified electrodes, using water-soluble carbodiimide and *N*-hydroxysulfosuccinimide reagents to activate carboxylate groups on the surface, yielded following optimal proportions: 4.5 % (w/w) stearic acid and 10 $\mu g \cdot mL^{-1}$ DNA. Polythymidylic acid of 4,000-base average length (poly $(dT)_{4000}$) was immobilized at stearic acid-modified electrodes following enzymatic elongation with dG residues at the 3'-end. These DNA-modified electrodes were used to study hybridization with analyte poly$(dA)_{4000}$ by *in situ* voltammetry of 60 μM $Co(bpy)_3(ClO_4)_3$ at low ionic strength (20 mM NaCl), and by voltammetry of same complex, following exposure of the electrode to poly$(dA)_{4000}$ in a separate hybridization step conducted at high ionic strength (0.5 M NaCl). Results indicated slow (≥1 h) hybridization at low ionic strength and fast (≤10 min) hybridization at high ionic strength. At high ionic strength, a detection limit of 2.5 ng of poly$(dA)_{4000}$ was obtained, which corresponded to 250 pg of a typical 400-bp polymerase chain reaction product. The results were applied to the selective detection of the cystic fibrosis ΔF508 sequence in an 18-base oligodeoxynucleotide sample.

Apparently electrochemical transduction-based DNA sensors are easy to construct, needing only two electrodes and a voltammeter; however, the sensitivity and specificity of such constructs is poor.

DNA BIOSENSORS BASED ON PIEZOELECTRIC TRANSDUCERS

Piezoelectric materials have been used in a variety of configurations as microgravimetric detectors, and their general theory and use has been well reviewed [3,4,46]. They offer an attractive, near-universal mode of transducing the biorecognition event, but only if the changes in detector mass that accompany analyte binding are sufficiently large. Resolution of mass changes to $<1\times10^{-9}$ $g \cdot cm^{-2}$ is possible in liquid media and, at least for high molecular mass substances, this provides for a viable transduction strategy. DNA biosensors using piezoelectric crystals have been developed more extensively [25,36–39,47–51] compared to other types of transducers.

Piezoelectric transducers also offer the advantages of a solid-state construction, chemical inertness, durability, and ultimately the possibility of low cost mass production. Attention to date has been mainly on AT-cut quartz crystals as the piezoelectric material that can function in a 'microbalance' mode. In order to carry out a measurement, an external voltage is used to deform the quartz crystal plate so that there is relative motion between the two parallel crystal surfaces (thickness shear), crystal relaxation and oscillation at the

resonant frequency then being maintained by means of an appropriate external circuit. The change in frequency (Δf) resulting from any added mass (Δm) to the device can be described by the Sauerbrey equation [52]. Thus, at least to a first approximation, the change in mass per unit area of the crystal is directly proportional to the change in frequency. A number of basic assumptions underlie this relation, however, and various modifying theories have been proposed to account for deviations in both gas- and liquid-phase operation [46].

DNA sequences of even a few hundred base pairs have considerable molecular weights. It seems likely, therefore, that the mass change associated with DNA hybridization might be detectable by employing piezoelectric devices. Fawcett *et al.* accomplished this by immobilizing ssDNA onto quartz crystals and detecting the mass change after hybridization. Nucleic acid strands were covalently attached to the polymer-modified surface of a piezoelectric crystal. When these immobilized probe strands were melted and then incubated with complementary target strands in solution, association of probe and target to form duplexes resulted in an increase in mass that was detectable as a decrease of several hundred hertz in the crystal's resonance frequency relative to control crystals on which non-complementary strands were attached. Those encouraging results suggest that the method may be useful as a non-radioactive means of identifying DNA and RNA. Furthermore, the crystals used are inexpensive, and the results are quantitative as well as qualitative. Similar results were also achieved in another study [36]. The hybridization assays were performed in solution, but the frequency measurements were performed in the dry state.

Mass detection with piezoelectric material can also be achieved using the principle of the surface acoustic wave (SAW). In such devices, an interdigitated array of electrodes in the material generates local deformations that are transmitted as mechanical waves to a receiver electrode array [46]. Interaction of the generated wave with any surface material alters SAW speed and amplitude, hence enabling quantification of the deposited mass. Su *et al.* [25,38,39] described the application of acoustic wave techniques to DNA biosensors. Single-strand DNA labeled with ^{32}P by the random primer method was adsorbed to the surface of palladium electrodes of thickness shear-mode acoustic wave sensors and nylon membranes under various solution conditions. The mass of nucleic acid attached to these substrates was quantified by scintillation count and phosphor image analysis. Measurement of the mass on sensors by acoustic wave sensor response obtained in the gas phase did not correlate with the values obtained by radiochemical labeling. Hybridization of cDNA to the single-strand species on the sensor surface was also determined by the ^{32}P technique. The added mass could be correlated with real-time changes in the series resonant frequency obtained *in situ* from acoustic network analysis. The frequency was altered 18 times the value predicted by the Sauerbrey expression. This behavior was ascribed to changes in interfacial viscosity originating from duplex formation. The limit of detection of the unoptimized technique was 2 ng of on-surface added nucleic acid. In addition to the series

resonant frequency, other parameters from network analysis, such as the motional resistance, have been measured on a real-time basis during interfacial hybridization. This technique has been applied to the determination the concentration of an anticancer drug binding to DNA [25]. Nucleic acid was attached to the electrodes of thickness-shear-mode acoustic wave devices to produce a biosensor for the platinum-based drugs. The decreases in series resonant frequency for interactions of DNA with both cis- and transplatin were indicative of two distinct kinetic processes. The results of a kinetic analysis were interpreted in terms of nucleic acid binding of the hydrolysis products of the two drugs. Concentration-dependent decreases of series resonant frequency showed that the limit of detection for the drugs was approximately 10^{-7} M. Motional resistance changes for nucleic acid-drug interactions also conveyed information about the chemistry of the macromolecules at the interface.

The mechanism of liquid phase operation of piezoelectric devices remains uncertain for some conditions [53]. In the liquid phase, sensitivity is reduced due to dampening of the measured wave component normal to the surface. Recent research has centered on the role of interfacial parameters such as surface viscosity, free energy and morphology in determining the response, and particularly the series resonant frequency, of the thickness-shear-mode (TSM) devices in the liquid phase [54–56]. This technique has been employed to examine nucleic acid hybridization at the sensor-solution interface in attempts to build a DNA biosensor [37,38]. The series resonant frequency change was related to the DNA hybridization process through the DNA concentration-dependent viscosity change in the acoustic wave decay length region [38]. The kinetics of hybridization of pPT-2 cDNA probe to ssDNA immobilized on thickness-shear-mode (TSM) acoustic wave sensors have been examined. The process as measured by this device did not conform to the generally assumed theoretical second order reaction in solution and one-step mechanism on a typical membrane filter. The pseudo-first order effective rate constants were $(8.0 \pm 0.5) \times 10^{-5}$ s^{-1}. The calculated Mark-Houwink constant, α, was 1.8, and the intrinsic viscosity was 9×10^{4} (M^{-1}) under the hybridization conditions. Inflection points due to the formation of intermediate hybrids were observed during continuous measurements of series resonant frequency with time.

CONCLUDING REMARKS

As indicated above, over the last ten years, there has been a great surge in DNA biosensor research. Much of this work has focused on fiber optic, electrochemical, and piezoelectric transducers. Optical transducer-based biosensors are receiving increasing attention. BIA analysis, based on surface plasmon resonance (SPR) technique, perhaps offers the most reliable and desirable tool for DNA biosensor configuration.

A pretreatment process is often needed to detect a sample with a DNA biosensor because single-stranded oligonucleic acid probes can only hybridized to their

complementary sequence: single-stranded target DNAs. Initially, release of nucleic acid from samples is required by mechanical [57], thermal, chemical (SDS), or biological (enzyme) methods. Then nucleic acids must be denatured using thermal or other measures. When only very low concentrations of target nucleic acids is available, a well-developed technique, the PCR might be used to amplify the target DNA to a level that is suitable for biosensor detection.

With the recent advent of DNA probe technology, a number of selective oligomers which interact with the DNA of important biological species have been identified. These have been used to provide a new type of selective biorecognition element which is highly selective, stable, and can be easily synthesized in the laboratory as compared to other chemically synthesized biorecognition elements, such as catalytic antibodies. As a result, species-specific DNA probes may now be exploited for biosensor development. It usually takes about 20 hours or more to finish a traditional filter hybridization procedure [21], thus, DNA biosensing is often thought of as slow. However, great effort has gone into reducing the time of DNA hybridization. As noted in this review, many DNA biosensors will now produce results within an hour [27,29–31,33]. Nevertheless, a simple, fast, and inexpensive DNA based biosensor suited to routine use still remains to be developed. Considering the high chemical stability of DNA relative to other recognition elements such as enzymes, antibodies or other selective proteins, we remain optimistic about the near-term possibility of a truly rapid, sensitive, easy-to-use and disposable DNA biosensor.

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